De Novo Ceramide Synthesis Participates in the Ultraviolet B Irradiation-Induced Apoptosis in Undifferentiated Cultured Human Keratinocytes

Yoshikazu Uchida*, Anna Di Nardo*‡, Vanessa Collins*, Peter M. Elias*, and Walter M. Holleran*†

*Dermatology Service and Research Unit, Department of Veterans Affairs Medical Center and Department of Dermatology, School of Medicine, and †Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA, USA and ‡San Gallicano Dermatologic Institute, Rome, Italy.

Ultraviolet irradiation is a major environmental cause of skin cancers, whereas ultraviolet-induced DNA repair and apoptosis are defense mechanisms that rescue and/or protect keratinocytes from this risk. Multiple pathways are involved in ultraviolet-induced keratinocyte apoptosis, including activation of p38-mitogen-activated protein kinase, protein kinase C, and CD95, each of which are associated with caspase activation. Alternatively, ceramides could serve as ultraviolet-induced, second messenger lipids, because they induce cell cycle arrest and apoptosis in a variety of cell types, including keratinocytes. We investigated the role of ceramide *versus* caspase, and the responsible pathway for ceramide generation in ultraviolet B-induced apoptosis of cultured normal human keratinocytes maintained in low calcium (0.07 mM) medium. Ultraviolet B (40 mJ per cm^2) significantly inhibited cultured normal human keratinocyte proliferation, assessed as [^{3}H-methyl]thymidine-thymidine incorporation into DNA, 2 h after irradiation. Terminal nick deoxynucleotide end-labeling-positive apoptotic cells (14.8% at 24 h and 34.4% at 48 h) and trypan blue-positive apoptotic cells (8.4% at 24 h and 28.6% at 48 h) became evident in a time-dependent manner after ultraviolet B irradiation, in parallel with activation of caspase-3. The ceramide content of irradiated cultured normal human keratinocytes increased significantly by 8 h, whereas glucosylceramide only modestly increased, and sphingomyelin content remained unaltered. Metabolic studies with radiolabeled serine, palmitic acid, and phosphorylcholine revealed that the ultraviolet B-induced increase in ceramide results primarily from increased *de novo* synthesis rather than accelerated sphingomyelin hydrolysis. Increased ceramide synthesis, in turn, could be attributed to increased activity of ceramide synthase (i.e., 1.7-fold increase 8 h after ultraviolet B irradiation), whereas serine palmitoyltransferase activity did not change. Both fumonisin B1, an inhibitor of ceramide synthase, and ISP-1, myriocin an inhibitor of serine palmitoyltransferase, significantly attenuated the ultraviolet B-induced apoptosis in a caspase-3-independent fashion, whereas co-incubation with a caspase-3 inhibitor (Ac-DEVD-chloromethyl-ketone) further attenuated the ultraviolet B-induced apoptosis. Thus, increased *de novo* ceramide synthesis signals ultraviolet B-induced apoptosis, by a pathway independent of, but in concert with, caspase-3 activation. ***Key words: apoptosis/ceramides/keratinocytes/sphingolipids/ultraviolet radiation. J Invest Dermatol 120:662–669, 2003***

Ultraviolet (UV) irradiation is a major cause of epidermal inflammation, immunosuppression, altered epidermal permeability barrier function, premature aging, dyspigmentation, and the development of both nonmelanoma and melanoma skin cancers (Fisher *et al*, 1996; Gilchrest *et al*, 1996; Haratake *et al*, 1997a, b; Kraemer, 1997; Beissert and Schwarz, 1999). Although UVC and shorter wavelengths of UVB (<295 nm) are filtered by the ozone layer, the remaining UVB (>295 nm) and UVA reach the earth's surface, posing a significant risk of dose-dependent toxicity. Whereas photocarcinogenesis is the hazard of greatest concern for skin, UV-induced cell cycle arrest, coupled with DNA repair and/or apoptosis, represent potential rescue mechanisms that eliminate UV-induced, mutated gene(s) from the epidermis (Brash *et al*, 1996).

Previous studies have demonstrated that multiple signaling pathways are associated with UV-induced apoptosis. Direct activation of CD95, independent of CD95 ligand, by UVB causes cell death in a spontaneous transformed keratinocyte cell line (HaCaT) (Aragane *et al*, 1998). UVB or UVC irradiation also activate caspases that induce keratinocyte apoptosis (Schwarz *et al*, 1995; Leverkus *et al*, 1997; Rehemtulla *et al*, 1997; Aragane *et al*, 1998). In addition, protein kinase C δ activation by proteases of the caspase family is involved in UVB-induced apoptosis in cultured normal human keratinocyte (CHK) (Denning *et al*, 1998). Moreover, p38 mitogen-activated protein kinase activation

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Reprint requests to: Walter M. Holleran, Pharm.D., Dermatology Service & Research Unit (190), Department of Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121, U.S.A. Email: walth@itsa.ucsf.edu

Abbreviations: CHK, cultured human keratinocytes; SPT, serine palmitoyltransferase; HPTLC, high-performance thin layer chromatography; UV, ultraviolet.

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of caspases results in apoptosis in CHK exposed to UVB (Shimizu *et al*, 1999); however, the UVB induced p38 mitogen-activated protein kinase-stimulated cytochrome *c* release from mitochondria occurs via a caspase-independent mechanism in CHK (Assefa *et al*, 2000).

Over the past decade, elevated cellular ceramide levels have been linked to cellular stress resulting from increased levels of reactive oxygen species, cytokines, exposure to chemotherapeutic agents, irradiation, exogenous lipopolysaccharides, etc. (reviewed in: Mathias *et al*, 1998; Hannun and Luberto, 2000; Andrieu-Abadie *et al*, 2001). Increased ceramide, in turn, provokes cell cycle arrest and/or apoptosis in a variety of cell types. Stressors increase ceramide either by (i) acceleration of sphingomyelin hydrolysis resulting from activation of sphingomyelinase (Peña *et al*, 1997; Billis *et al*, 1998; Singh *et al*, 1998; Hernandez *et al*, 2000), and/or (ii) increased *de novo* ceramide synthesis, through activation of either ceramide synthase (Bose *et al*, 1995; Xu *et al*, 1998; Garzotto *et al*, 1999; Liao *et al*, 1999) or serine palmitoyltransferase (SPT) (Lehtonen *et al*, 1999; Herget *et al*, 2000; Perry *et al*, 2000). The downstream targets from such stress-induced increases in ceramide include ceramide activated protein kinase, stress-activated protein kinase/c-Jun N-terminal kinase, protein kinase C-α/ζ ceramide activated phosphatase, cathepsin D, phospholipase A_2, phospholipase D, and nuclear factor-κB cells, depending on tissue type (Mathias *et al*, 1998; Perry and Hannun, 1998; Hannun and Luberto, 2000; Andrieu-Abadie *et al*, 2001).

Keratinocytes are one of several cell types that are susceptible to ceramide challenge (Geilen *et al*, 1997, 2001). In fact, some studies have demonstrated that alterations in cellular ceramide level from either exogenous short chain ceramide (C2–C8 ceramide), or exogenous bacterial sphingomyelinase inhibit DNA synthesis (Wakita *et al*, 1994; Uchida *et al*, 2002), and induce CHK apoptosis (Iwasaki-Bessho *et al*, 1998; Wieder *et al*, 1998; Di Nardo *et al*, 2000). We also demonstrated that SPT is upregulated transcriptionally and ceramide synthesis is increased in CHK exposed to UVB (Farrell *et al*, 1998). Although Shimizu *et al* (1999) concluded that sphingomyelin hydrolysis to ceramide is not involved in the HaCaT keratinocyte response to UVB, a recent study also indicates that the stress/apoptosis signaling pathway in HaCaT cells differs from that in CHK (Chaturvedi *et al*, 2001). As such, the involvement of *de novo* synthesized ceramide in UVB-induced CHK cell-cycle arrest and apoptosis has not been adequately addressed. In this study, we directly examined whether ceramide-mediated events are responsible, at least in part, for UVB-induced CHK apoptosis. We demonstrate that ceramide synthesis is activated early following UVB irradiation, an effect attributable to increased ceramide synthase activity, and that blockade of *de novo* ceramide synthesis inhibits the UVB-induced apoptosis. In addition, the ceramide-associated cell death pathway in response to UVB represents a caspase-3-independent apoptotic pathway in CHK.

MATERIALS AND METHODS

Chemicals Ceramides, glucosylceramides, sphingomyelin, fumonisin B1, *N*-acetyl-Asp-Glu-Val-Asp-amido-4-methyl-coumarin (Ac-DEVD-AMC), and *N*-acetyl-DEVD-aldehyde (Ac-DEVD-CHO) were purchased from Sigma (St Louis, MO). ISP-1 was from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). Myriocin Ac-DEVD-chloromethyl-ketone (Ac-DEVD-CMK) was from Calbiochem (San Diego, CA). Radiolabeled chemicals were from American Radiolabeled Chemicals Inc. (Arlington Heights, IL). High-performance thin-layer chromatography (HPTLC) plates (Silica Gel 60) were purchased from Merck (Darmstadt, Germany).

Cell culture Normal human keratinocytes were isolated from human neonatal foreskins by a modification of the method of Pittelkow and Scott (1986). Cells were grown in keratinocyte grown medium, supplemented with bovine epidermal growth factor, bovine pituitary extract, insulin, hydrocortisone, and 0.07 mM calcium (Cascade Biologics, Portland, OR). Increasing calcium concentration stimulates keratinocyte differentiation and alters lipid metabolism, including sphingolipids (Ponec *et al*, 1988). Although fully differentiated keratinocytes generate a complex and heterogeneous group of ceramide and glucosylceramide molecular species (Lampe *et al*, 1983a, b; Wertz and Downing, 1983a, b), some of which have been implicated in promoting keratinocyte differentiation (Uchida *et al*, 1990), the involvement of these more complex sphingolipid species in cell cycle arrest, differentiation, and/or apoptosis remains unknown. As less-differentiated keratinocytes generate a limited number of ceramide and glucosylceramide species (Ponec *et al*, 1988), we have employed monolayer-undifferentiated cells cultured in 0.07 mM calcium for these studies. The cultures were maintained at 37°C under 5% CO_2 in air, with medium changes performed three times weekly.

UVB irradiation UVB irradiation was performed as described previously (Farrell *et al*, 1998). Briefly, cells were seeded ($2-4 \times 10^4$ cells per ml) in two well glass chamber slides, 12 multiwell plates, 60 mm or 100 mm dishes and maintained to 80–100% confluence (monolayer cultures). The cells then were rinsed with phosphate-buffered saline containing 0.07 mM calcium, and treated with UVB (emission range 280–340 nm 305 nm max, FS 20/T12 bulbs, National Biological Co., Twinsburg, OH) in phosphate-buffered saline. UVB exposure was measured using a Goldilux Ultraviolet Radiometer (Oriel, Stratford, CT). Cells were exposed to a single dose of UVB (60 mJ per cm^2) in most studies, unless indicated otherwise. Immediately after UVB irradiation, phosphate-buffered saline was replaced by culture medium, with or without an added sphingolipid synthetic inhibitor; i.e., fumonisin B1 or ISP-1.

Cellular proliferation Total cellular DNA was determined by the method of Labarca and Paigen (1980), using the fluorescent reagent, bis-benzimidazole. Keratinocyte growth was assessed as [^{3}H-methyl] thymidine incorporation into DNA, as described previously (Farrell *et al*, 1998). At appropriate time points following UVB treatment, cells were incubated with 1 μCi per ml of [^{3}H-methyl]-thymidine for 1 h at 37°C, and the quantity of label in trichloroacetic acid-precipitable macromolecules was determined by liquid scintillation spectrometry. DNA synthesis data are expressed as methyl-[^{3}H]-L-thymidine incorporated per mg DNA.

Assays for apoptosis Apoptotic cells were assessed both by terminal deoxynucleotidyltransferase-mediated digoxigenin-deoxyribonucleotide nick-end labeling (TUNEL assay) and by trypan blue-dye exclusion. TUNEL staining was performed using the ApopTag Apoptosis Detection Kit, following the manufacturer's protocol (Intergen Co., Purchase, NY). At least 200 cells were chosen at random on each slide to quantitate apoptosis. As a further measure of apoptosis, caspase-3 activities in cell lysates were determined using a modification of the method of Nicholson *et al* (1995). Briefly, cells were washed with ice-cold phosphate-buffered saline, incubated with 50 mM HEPES buffer, pH 7.4, containing 5 mM CHAPS, 5 mM dithiothreitol, and protease inhibitors (Protease Inhibitor Cocktail for Mammalian Cell and Tissue Extracts, Sigma), for 30 min on ice, followed by separation of supernatants by centrifugation ($14{,}000 \times g$ at 4°C). Fifty micrograms of protein was incubated with Ac-DEVD-AMC with or without addition of the caspase-3 inhibitor, N-acetyl-Asp-Glu-Val-Asp-al (Ac-DEVD-CHO), for 30 min at 30°C. Fluorescent product (AMC) was measured by fluorescence spectrophotometry, and activities are reported as AMC generated per min per mg of protein. Protein content was determined by the BCA protein assay method (Pierce, Rockford, IL), using bovine serum albumin as the standard.

Lipid analysis Total lipids were extracted from CHK by the method of Bligh and Dyer (1959), separated into individual lipid species by HPTLC, followed by quantification by scanning densitometry, as described previously (Holleran *et al*, 1991). To assess sphingomyelin hydrolysis following UVB irradiation, cells (preconfluent) were incubated with [^{3}H-methyl]choline chloride (1 μCi per ml) or [^{3}H]serine (2.5 μCi per ml) for 2 d followed by incubation in a radioisotope-free medium for 16 h. Alternatively, to examine *de novo* lipid synthesis, cells were cultured with [^{3}H]serine (1.5 μCi per ml) or [9,10-^{3}H]palmitic acid (1.5 μCi per ml) for the final 3 h. Lipids were extracted and analyzed, as described previously (Farrell *et al*, 1998), and radioisotope incorporated into each lipid fraction was measured by liquid scintillation spectroscopy.

Ceramide synthase assay The assay employed to measure ceramide synthase activity was a modification of that of Bose *et al* (1995). Briefly, microsomal protein (75–100 μg) was incubated with 18 μM sphinganine and 70 μM [1-^{14}C]palmitoyl-coenzyme A (spec. act. 10–15,000 dpm per nmol) at 37°C (60 min). The reaction product, palmitoylsphinganine, was extracted and isolated by HPTLC. The activity was expressed as palmitate equivalents incorporated into sphinganine per min per mg protein.

SPT activities SPT was assayed as previously described (Holleran *et al*, 1990; Weiss and Stoffel, 1997). Microsomal protein (50–75 μg) was incubated with 50 μM pyridoxal phosphate, 300 μM palmitoyl-coenzyme A, 1.0 mM [G-^{3}H] L-serine (spec. act. 45–50,000 dpm per nmol) at 37°C (15 min). The reaction product, 3-ketodihydrosphinganine was then reduced to sphinganine using sodium borohydride and extracted with chloroform–methanol (4:1, v/v) followed by isolation of sphinganine by HPTLC. Radioisotope incorporated into the sphinganine fraction was measured as described above, and SPT activity is expressed as sphinganine produced per min per mg protein.

Statistical analysis Statistical analyses were performed using an unpaired Student's t test.

RESULTS

UVB irradiation inhibits DNA synthesis and induces apoptosis in CHK To understand how CHK respond to UVB, we first examined the effect of UVB irradiation on DNA synthesis in CHK. Cells were exposed to 0–100 mJ per cm^2 UVB, and thymidine incorporation into DNA synthesis was measured 2–24 h following irradiation. UVB irradiation inhibited DNA synthesis significantly in a dose-dependent manner (**Fig 1*A***), an effect that began within 2 h after irradiation, and was maintained during the entire 24 h period (**Fig 1*B***).

We next assessed the extent of apoptosis induced by UVB, using three independent markers: TUNEL staining, trypan blue exclusion, and caspase-3 activity. TUNEL-positive, apoptotic cells became evident 24 h following UVB treatment, increasing by 48 h in cells irradiated with 60 mJ per cm^2 (**Fig 2*A***), but apoptosis was not evident in cells irradiated at doses below 40 mJ per cm^2 (data not shown). As cells become permeable to trypan blue during the later phases of apoptosis, we also assessed apoptotic cells using the trypan blue exclusion assay (van Heerde *et al*, 2000). Consistent with the

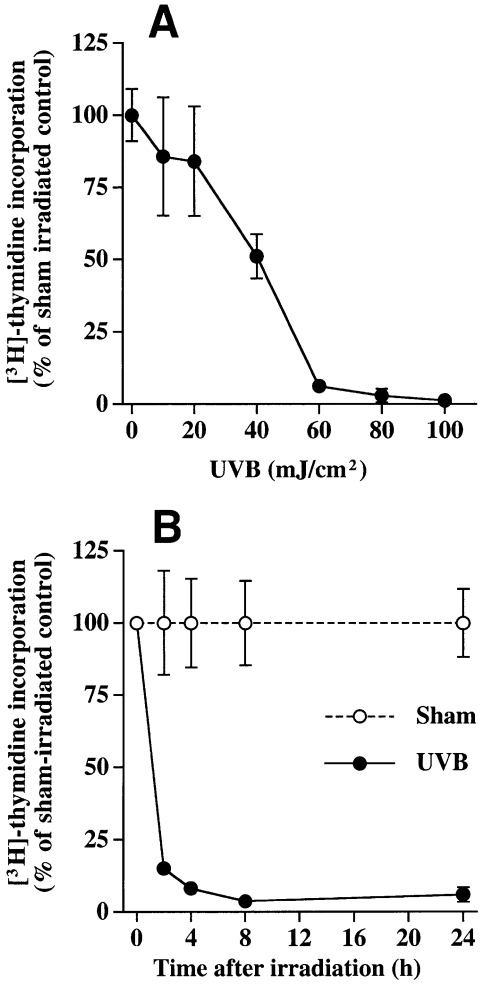

Figure 1. UVB irradiation inhibits DNA synthesis of CHK. CHK were incubated for 24 h after a single dose of UVB (0–100 mJ per cm^2) (*panel A*), or for 2–24 h after a single UVB dose (60 mJ per cm^2) (*panel B*). Data are expressed as a percentage (mean ± SEM) of sham-irradiated control cells. The inhibitory effect of a 60 mJ per cm^2 dose was evident by 2 h after treatment (*panel B*), and doses ⩾60 mJ per cm^2 induced complete inhibition of CHK proliferation at 24 h (*panel A*); *$p < 0.001$ for all time points (2–24 h) *vs* sham-irradiated control cells; n = 12.

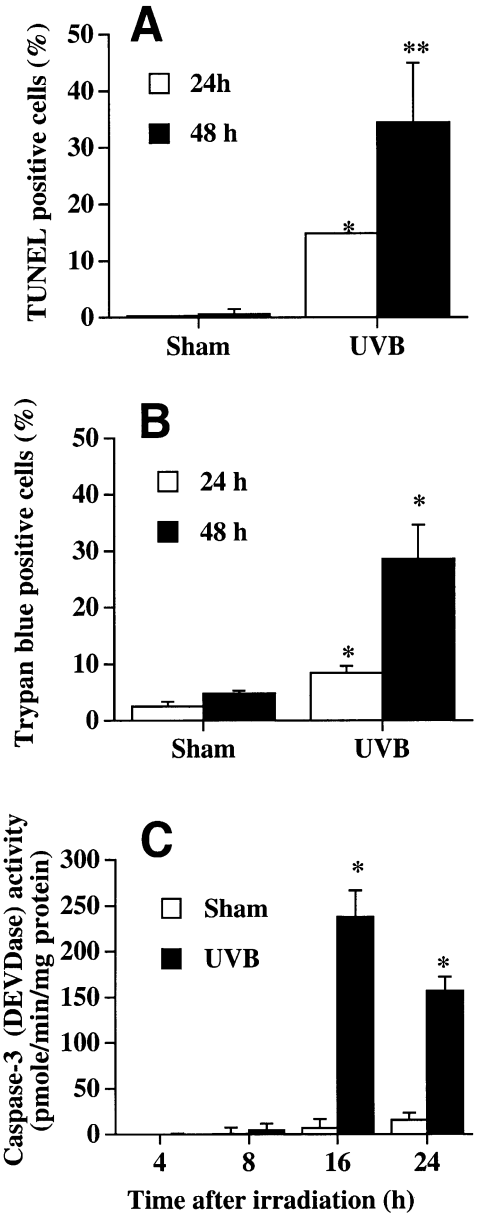

Figure 2. UVB irradiation-induced apoptosis in CHK. CHK were incubated for 24 or 48 h following single UVB exposure (60 mJ per cm^2). Apoptosis was assessed by TUNEL staining (*panel A*); by trypan blue dye exclusion assay (*panel B*); and by caspase-3 assay (*panel C*). TUNEL- and trypan blue-positive cells are reported as a percentage of total cells; caspase activity is expressed as pmol per min per mg protein. In each case, data are reported as mean ± SEM (*panels A,B,* n = 6; *panel C,* n = 3). TUNEL-positive and trypan blue-positive cells were evident by 24 h, and further increased at 48 h (*panels A,B*), whereas caspase-3 activity was increased at 16 h, and remained elevated at 24 h (*panel C*); *$p < 0.001$ and **$p < 0.01$ *vs* sham-irradiated control cells, respectively.

TUNEL assay, the proportion of trypan blue positive cells also increased following UVB irradiation (60 mJ per cm^2) in a time-dependent manner (**Fig 2*B***). Finally, activation of caspase-3, a key effector enzyme of apoptosis, became evident 16–24 h after UVB treatment (**Fig 2*C***). These results indicate that UVB irradiation of CHK causes a rapid decline in DNA synthesis, followed by induction of apoptosis.

Ceramide content increases in UVB-irradiated CHK Acute cellular stress, e.g., from reactive oxygen species or inflammatory cytokines, is associated with increased cellular ceramide levels (Mathias *et al*, 1998; Perry and Hannun, 1998; Andrieu-Abadie *et al*, 2001). We next studied the effects of UVB irradiation on sphingolipid levels in CHK. Ceramide content increased significantly with time after UVB irradiation (i.e., 1.3–1.9-fold *vs* sham-irradiated controls, from 4 to 48 h, respectively) (**Fig 3**). Neither ceramide or glucosylceramide levels changed in control cells (i.e., ceramide: 10.3–13.2 μg per mg DNA and glucosylceramide 10.1–11.4 μg per mg DNA) at all time points. The glucosylceramide content of CHK, however, also increased significantly (1.2-fold) in irradiated control cells ($p < 0.02$), an increase that was smaller than the change in ceramide after irradiation (**Fig 3**). In contrast, the content of sphingomyelin in CHK (control cells: 212–244 μg per mg DNA at 4–48 h) did not change significantly after UVB irradiation (**Fig 3**). These results indicate that substantial alterations of ceramide levels occur following exposure of CHK to UVB, and suggest further that this increase is not due to increased sphingomyelin degradation.

UVB irradiation stimulates *de novo* synthesis of ceramide To investigate the mechanism (i.e., increased sphingomyelin hydrolysis or increased ceramide synthesis) responsible for the increase in ceramide levels in UVB-irradiated CHK, we first examined sphingomyelin hydrolysis using cells prelabeled with either [^{3}H]serine or [^{3}H]choline. Sphingomyelin levels did not decrease significantly between 0.25 and 8 h after irradiation (data not shown), time points during which ceramide content was increasing (see above). As the UVB-induced increase in ceramide content could not be attributed to increased sphingomyelin hydrolysis, we next examined the effects of UVB on *de novo* sphingolipid synthesis in CHK. Ceramide synthesis assessed as [^{3}H]palmitate incorporation into sphingolipids increased significantly at 8 h after 60 mJ per cm^2

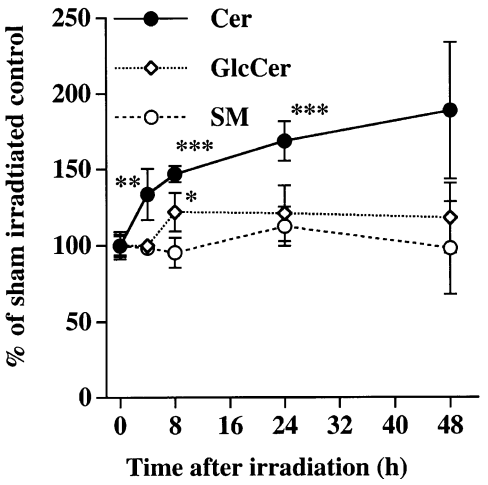

Figure 3. UVB irradiation increased ceramide content of CHK (60 mJ per cm^2). Data for ceramide (closed circles), glucosylceramide (open rhombus), and sphingomyelin (open circles) content are expressed as a percentage (mean ± SEM) of sham-irradiated controls. UVB induced a significant increase in total ceramide content at 8 and 16 h. Increased glucosylceramide content was evident at 8 h, whereas sphingomyelin content remained unchanged throughout the treatment period; $^*p < 0.02$, $^{**}p < 0.01$, and $^{***}p < 0.001$ each *vs* sham-irradiated control values; n = 4.

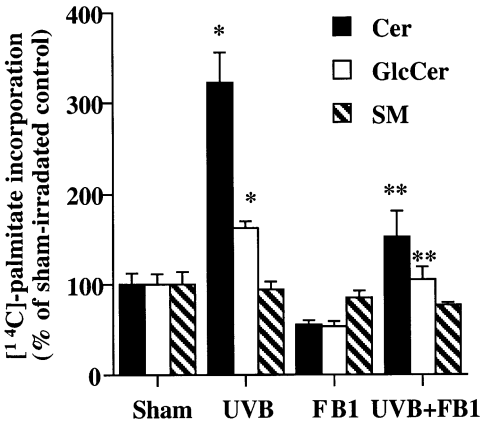

Figure 4. UVB irradiation increases *de novo* ceramide and glucosylceramide, but not sphingomyelin, synthesis. CHK were incubated with [^{14}C]palmitate for 8 h following UVB irradiation (60 mJ per cm^2). Fumonisin B1 (FB1) was added immediately after UVB or sham treatment. Data are expressed as a percentage (mean ± SEM) of sham-irradiated controls. The UVB-induced increases in ceramide and glucosylceramide synthesis ($^*p < 0.005$) were significantly inhibited by fumonisin B1; $^{**}p < 0.01$ *vs* UVB treated cells without fumonisin B1; n = 4.

UVB treatment (3.2-fold, $p < 0.005$ *vs* control) (**Fig 4**). Glucosylceramide synthesis also increased significantly (1.6-fold, $p < 0.005$ *vs* control), whereas *de novo* synthesis of sphingomyelin did not change. Furthermore, treatment of cells with fumonisin B1 (50 μM), an inhibitor of ceramide synthase (Merrill *et al*, 1993), attenuated the UVB-induced increase in both ceramide (47.2% of UVB alone, $p < 0.01$) and glucosylceramide (64.4% of UVB alone, $p < 0.01$) synthesis, further evidence that the UVB-induced ceramide increase is due to stimulation of *de novo* ceramide synthesis.

To assess the basis for the increase in ceramide synthesis, we next assayed changes in the activities of two key enzymes of ceramide synthesis, ceramide synthase and SPT, in UVB-irradiated CHK. Ceramide synthase activity increased at 4 h (1.2-fold; $p < 0.02$) and 8 h (1.7-fold; $p < 0.01$) following UVB treatment (**Table I**). In contrast, SPT activity did not change following irradiation (data not shown). In addition, [^{3}H]serine incorporation into the ceramide fraction in CHK did not increase following UVB irradiation (data not shown), a further indication that SPT activity remains unchanged under these conditions. These studies demonstrate that the increase in ceramide generation after UVB irradiation reflects increased N-acylation of sphingoid base primarily due to increased ceramide synthase activity.

Blockade of *de novo* ceramide synthesis decreases UVB-induced apoptosis To investigate whether the UVB-induced increase in ceramide causes apoptosis in irradiated CHK, we next assessed whether inhibition of ceramide synthesis could attenuate the UVB-induced apoptosis. Fumonisin B1, at a concentration that significantly inhibited ceramide synthesis

Table I. Ceramide synthase activity increases following UVB irradiation

	% of Sham-irradiated control cells	
	4 h	8 h
Sham	100.0 ± 0.08	100.0 ± 1.1
UVB radiation	122.9 ± 3.1*	166.7 ± 14.1**

n = 3, mean ± SEM. $^*p < 0.02$, $^{**}p < 0.01$ *vs* sham-irradiated control cells.

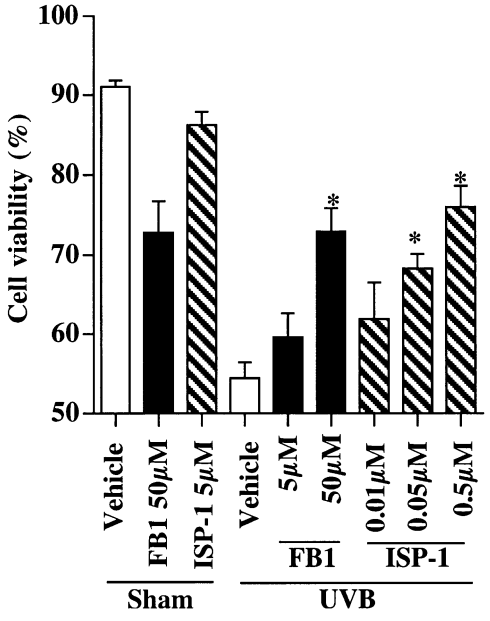

Figure 5. Both fumonisin B1 and ISP-1 decreased UVB-induced apoptosis in CHK. CHK were irradiated (60 mJ per cm^2) and incubated with or without inhibitors for 48 h. Cell viability/apoptosis were assessed by trypan blue dye exclusion (cf. **Fig 2**). Data are expressed as a percentage (mean ± SEM) of sham control values (note expanded axis). Both fumonisin B1 (50 μM) and ISP 1 (≥0.05 μM) significantly attenuated the deleterious effect(s) of UVB on CHK viability; *$p < 0.01$ *vs* UVB-treated cells without inhibitor(s); $n \geq 3$.

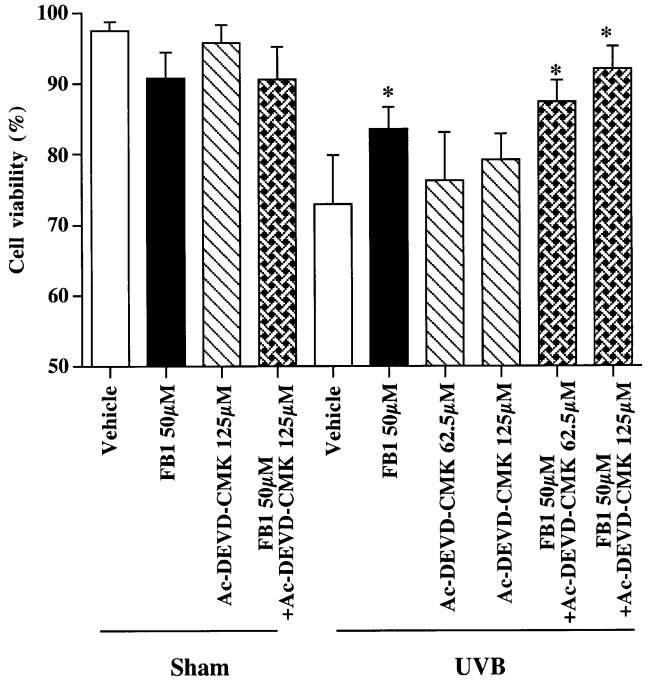

Figure 6. Co-administration of fumonisin B1 and caspase-3 inhibitor further decreased UVB-induced apoptosis in CHK. CHK were irradiated (60 mJ per cm^2) and incubated with or without fumonisin B1 (50 μM) for 42 h; the caspase inhibitor, Ac-DEVD-CMK, was added at the doses indicated (i.e., 62.5 or 125 μM) 2 h after irradiation. Cell viability was assessed by trypan blue exclusion (as above), and reported as a percentage (mean ± SEM) of non-UVB-treated sham control values (note expanded axis). Fumonisin B1 again significantly attenuates the UVB-induced decrease in cell viability. Although the effect of the caspase-3 inhibitor alone did not reach statistical significance, the combination of fumonisin B1 and Ac-DEVD-CMK showed further attenuation of the UVB-induced cell death; *$p < 0.01$ *vs* UVB-treated cells without inhibitors (i.e., 0/0 value).

(**Fig 4**), also significantly attenuated UVB-induced apoptosis, assessed by trypan blue-dye exclusion at 48 h, in a dose-dependent manner (i.e., 11.4% at 5 μM and 40.5% at 50 μM; $p < 0.01$ *vs* UVB alone) (**Fig 5**). The inhibitory effect of fumonisin B1 on UVB-induced apoptosis also was confirmed by TUNEL staining (data not shown). ISP-1, an inhibitor of SPT (Miyake *et al*, 1995), also decreased UVB-induced apoptosis significantly (i.e., by 30.2% and 47.2% at 0.05 μM and 0.5 μM, respectively; $p < 0.01$ *vs* UVB alone), indicating that, although increased SPT activation did not occur in UVB-treated cells, some newly synthesized sphingoid base is utilized for synthesis of the ceramide pool that causes apoptosis. Yet, despite 53% of inhibition of *de novo* ceramide synthesis, more than 30% of cells did not escape apoptosis when treated with optimal concentrations of fumonisin B1 or ISP-1, suggesting that ceramide-independent pathways also contribute to UVB-induced apoptosis in CHK.

UVB-induced caspase-3 activation is not inhibited by fumonisin B1 As noted above, the early ceramide induction (i.e., 3 h, **Figs 3 and 4**) occurs prior to caspase-3 activation (16–24 h, **Fig 2C**) following UVB irradiation. Moreover, some apoptosis occurs (approximately 30%), even when ceramide synthesis is largely blocked (**Fig 5**). Hence, we next studied the relationship between ceramide accumulation and activation of caspase-3 in UVB-treated CHK. To do so, we compared the effect(s) of fumonisin B1 and/or Ac-DEVD-CMK, an inhibitor of caspase-3, on UVB-induced apoptosis in CHK. Whereas each inhibitor alone decreased apoptosis, the combination of both fumonisin B1 and Ac-DEVD-CMK blocked apoptosis in an additive manner (**Fig 6**). As the UVB-induced increase in caspase-3 activation was not attenuated by fumonisin B1 (data not shown), these results provide further evidence that UVB stimulation of ceramide-induced apoptosis occurs by a caspase-3-independent pathway in CHK.

DISCUSSION

Although UV irradiation is essential for the synthesis of vitamin D3 in skin (Holick *et al*, 1980), excess skin exposure to UVB causes a wide variety of negative effects, most notably carcinogenesis (Kraemer, 1997). Interestingly, keratinocytes have evolved several levels of defense against UV-induced carcinogenesis. First, UV-induced cell cycle arrest followed by DNA repair is a well-recognized mechanism for amelioration of DNA damage. Second, endogenous anti-oxidant mechanisms (e.g., reduced glutathione, superoxide dismutase, catalase, vitamin E, and vitamin C) reduce UV-induced reactive oxygen species generation (reviewed in Thiele *et al*, 2001). Finally, the formation of sunburn cells, or apoptotic keratinocytes, following UVB stress is another mechanism whereby defective keratinocytes are eliminated, and thus are unable to contribute to carcinogenesis (Brash *et al*, 2001). This third mechanism of UV-induced keratinocyte apoptosis involves multiple signaling pathways, including CD95 activation (Aragane *et al*, 1998), p38 mitogen-activated protein kinase (Shimizu *et al*, 1999; Assefa *et al*, 2000; Nakamura *et al*, 2001), and protein kinase C activation (Denning *et al*, 1998). Recent studies have demonstrated that ceramides, the backbone of membrane structural glycosphingolipids and phosphosphingolipids, are signal transducers of a variety of cell stressors (Mathias *et al*, 1998; Perry and Hannun, 1998; Hannun and Luberto, 2000). In addition, ceramide generated from either the hydrolysis of sphingomyelin (Haimovitz-Friedman *et al*, 1994; Santana *et al*, 1996) or *de novo* synthesis (Bose *et al*, 1995; Lehtonen *et al*, 1999; Herget *et al*, 2000; Perry *et al*, 2000), depending on the tissue and insult type, can induce apoptosis. This study demonstrates that *de novo*

synthesis of ceramide plays an important part in UVB-induced apoptosis in CHK.

Although the increase in *de novo* ceramide synthesis following UVB irradiation is attributable primarily to ceramide synthase activation, ceramide generation via SPT also contributes to UVB-induced apoptosis. Indeed, both ISP-1 (an inhibitor of SPT) and fumonisin B1 (an inhibitor of ceramide synthase) decreased the UVB-induced cell death. Thus, the sphingoid base that is utilized by ceramide synthase in response to UVB could derive from two distinct sources; i.e., either from a pre-existing pool or from increased *de novo* synthesis. Both epidermis and CHK demonstrate high levels of SPT activity in comparison with other tissues (Holleran *et al*, 1990), indicating basal SPT activity in CHK may supply a sufficient sphingoid base for ceramide, synthesis, even in the face of UVB stress. Thus, constitutive, high-capacity sphingoid base production appears to supply sufficient sphingoid base for increased ceramide synthesis under conditions of cellular stress.

The finding that sphingomyelin production and content remain largely unchanged following UVB (**Figs 3 and 4**) is consistent with the report of Shimizu *et al* (1999), in which UVB induced neither sphingomyelin turnover nor sphingomyelinase activities in HaCaT keratinocytes. In contrast, a recent study by Magnoni *et al* (2002), demonstrated that both neutral and acidic sphingomyelinase activity increased early following UVB (i.e., 15 min to 1 h), whereas ceramide content increased significantly 16–24 h; changes in sphingomyelin content were not reported. In this case, the significant difference between the early sphingomyelinase activation and delayed increases in ceramide content suggest that sphingomyelin hydrolysis does not contribute directly to the later increases in cellular ceramide levels. Thus, the pathophysiologic significance of the early sphingomyelinase activation in UVB-irradiated keratinocytes remains unresolved. In the present study, fumonisin B1 blocked both the UVB-induced apoptosis and the increased ceramide production (**Figs 4 and 5**). Therefore, any ceramide generated from sphingomyelin hydrolysis or from or other sources appears to require further hydrolysis to sphingoid base, followed by re-utilization of the sphingoid base for ceramide synthesis. Thus, although we cannot eliminate the possibility that brief alterations in sphingomyelin hydrolysis may be involved in the overall epidermal response to UVB, *de novo* ceramide production, including involvement of both ceramide synthase and serine palmitoyltransferase activities, appears to be one of the important determinants of the apoptotic pathway.

The increases in both ceramide synthesis and content reported here and by Magnoni *et al* (2002) also are distinct from Shimizu *et al* (1999), in which no change in ceramide levels was observed following UVB. A study by Chaturvedi *et al* (2001), indicated that HaCaT cells are more susceptible to stress-induced apoptosis, including UVB, compared with normal human keratinocytes, due to diminished nuclear factor-B activation. Thus, it is feasible that these distinct results may reflect a differential response between HaCaT cell line and normal keratinocytes. In addition, it is possible that the overall response to UVB is different between keratinocytes cultured in low calcium (0.07 mM), as presented here, *vs* the higher calcium (1.2 mM) used in Shimizu *et al* (1999). Our results also do not appear to generalize to other forms of UV-induced stress. For example, the generation of singlet oxygen by UVA increases cellular ceramide levels via increased sphingomyelin degradation, generated by a nonenzymatic mechanism (Grether-Beck *et al*, 2000). This UVA-induced ceramide generation peaks much earlier than the UVB-induced ceramide formation; i.e., only 0.5–2 h after UVA. In the present study, UVB-induced ceramide induction is not evident until at least 3 h after irradiation. In addition, the latter increased ceramide generation is inhibited by fumonisin B1, demonstrating the involvement of ceramide synthase in this process. Therefore, we conclude that *de novo* ceramide synthesis, rather than nonenzymatic sphingomyelin hydrolysis, represents the key mechanism leading to ceramide-dependent, UVB-induced apoptosis in CHK.

Caspases, a family of cysteine proteases, cleave target proteins that ultimately induce apoptosis. Caspases-2, -8, -9, and -10 are the apoptotic initiator enzymes that activate a family of apoptotic executioner enzymes, caspase-3, -6, and -7 (Zimmermann *et al*, 2001), with caspase-3 being particularly important for the regulation of the apoptotic effects. Exogenous cell-permeant ceramide (e.g., *N*-hexanoyl sphingosine) causes apoptosis in HL-60 cells via accelerating Bax translocation from cytosol to mitochondria followed by increased caspase activity (Kim *et al*, 2001). In addition, both hypoxia-induced neutral sphingomyelinase activation, and exogenous ceramide (*N*-acetyl sphingosine) increase caspase-3 activity in PC12 cells (Yoshimura *et al*, 1998). Moreover, inhibition of *de novo* ceramide production by fumonisin B1 decreases both caspase-3 activation and apoptosis of Ms-1 cells treated with inostamycin (Kawatani *et al*, 2000). Although these studies suggest that ceramide is upstream of caspase-3 activation pathway, the present study demonstrates that inhibition of ceramide generation by fumonisin B1 does not block caspase activation, even though an increase in cellular ceramide level occurs prior to the UVB-induced activation of caspase-3. Furthermore, neither the caspase-3 inhibitor (Ac-DEVD-CMK) nor fumonisin B1 alone could completely block UVB-induced apoptosis in CHK, whereas fumonisin B1 and the caspase-3 inhibitor together additively decrease apoptosis. These results suggest that ceramide-induced apoptosis after UVB irradiation could be attributed to caspase-3-independent signaling pathway. Interestingly, etoposide-induced apoptosis, which is also preceded by increased *de novo* ceramide synthesis, occurs through a caspase-independent mechanism (Perry *et al*, 2000). Thus, UVB irradiation could induce keratinocyte death by multiple pathways, including a novel caspase-independent mechanism, along with a classical caspase-dependent pathway. Activation of diverse pathways should contribute to a more efficient induction of apoptosis in epidermis exposed to UVB, thereby preventing transformation to malignant cells (**Fig 7**).

Numerous studies, including our own, have demonstrated that UVB irradiation inhibits cellular proliferation in mammalian epidermis as well as in cultured keratinocytes (Epstein *et al*, 1970; Petrocelli *et al*, 1996; Courtois *et al*, 1997; Haratake *et al*, 1997a, b; Nakamura *et al*, 2001). An increase in cellular ceramide also induces inhibition of mitogenesis in multiple types of cells. For instance, serum depletion induces cell cycle arrest (G_0/G_1 arrest) with increasing cellular ceramide (Dbaibo *et al*, 1995; Jayadev *et al*, 1995), and cell cycle arrest also occurs in cells treated with exogenous short chain ceramide (Bourbon *et al*, 2000; Di Nardo *et al*, 2000; Spyridopoulos *et al*, 2001). Such cell cycle arrest correlates with: (i) dephosphorylation of the retinoblastoma gene product (Lee *et al*, 1998); (ii) inhibition of Akt phosphorylation/activation (Bourbon *et al*, 2000); and (iii) downregulation of cyclin A expression (Spyridopoulos *et al*, 2001). Previous reports have demonstrated that UVB causes G_1 arrest of CHK (Denning *et al*, 1998). We showed here again that UVB inhibits DNA synthesis within 2 h after irradiation (**Fig 1**), a time point at which

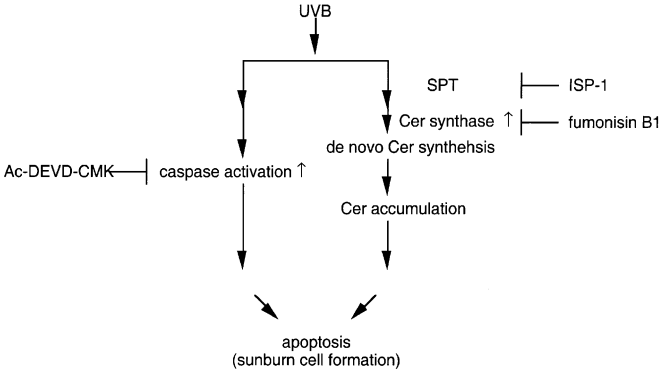

Figure 7. Proposed pathway of ceramide-induced apoptosis in CHK following UVB irradiation.

ceramide are not yet increased, indicating that the initial UVB-induced inhibition of DNA synthesis is not due to ceramide-mediated cell cycle arrest, but rather some other mechanisms (e.g., p53, Fas activation). It is clear from this study, however, that ceramide contributes to cell cycle arrest (as well as to the induction of apoptosis) at later time points following UVB; i.e., 8 h.

We report here that ceramide synthase, but not SPT, activity is increased in CHK treated with UVB (60 mJ per cm^2), resulting in elevated cellular ceramide levels leading to apoptosis. We previously demonstrated, however, that lower dose UVB (i.e., 23 mJ per cm^2) induced increased SPT activity in CHK via transcriptional regulation at later time points (Farrell *et al*, 1998). With the lower dose UVB, synthesis of total sphingolipids, including ceramide, glucosylceramide, and sphingomyelin, globally increase 24 h and 48 h after irradiation. In contrast to the higher dose of UVB used in this study, the lower UVB dose inhibited DNA synthesis at early (24 h) and restored/stimulated proliferation at later time points (48 h), whereas apoptosis was not evident in these cells. Similarly, suberythemal doses of UVB irradiation also increase ceramide content in human stratum corneum (Wefers *et al*, 1991). Therefore, keratinocyte ceramide synthesis responds differently to low *vs* high doses of UVB irradiation. Low-dose UVB stimulates sphingolipid synthesis by the activation of SPT, with a lag time of at least 24 h after irradiation, whereas high doses of UVB rapidly increase ceramide synthesis by activation of ceramide synthase. Although the biologic significance of increased sphingolipid synthesis following low-dose UVB is still not known, high-dose UVB irradiation may increase ceramide production as an additional mechanism to protect epidermis against photocarcinogenesis.

In vivo epidermis consists primarily of a spectrum of undifferentiated-to-differentiated keratinocytes. During differentiation, keratinocytes not only upregulate both ceramide and glucosylceramide synthesis, but also generate a heterogeneous molecular mixture of sphingolipid species (Lampe *et al*, 1983a, b; Wertz and Downing, 1983a, b). These unique ceramide and glucosylceramide species are localized primarily to lamellar bodies in the suprabasal, differentiating cells, and are destined for secretion into extracellular domains of the stratum corneum, where they subserve barrier function. Although it remains to be determined whether these epidermal-specific ceramide species are involved in the apoptotic response to UVB, this study demonstrates that increasing *de novo* synthesis, at least in undifferentiated keratinocytes, plays an important part in the UVB-induced events.

In summary, high-dose UVB increases *de novo* ceramide synthesis in parallel with the activation of ceramide synthase. This ceramide induction results in increased apoptosis in CHK via a caspase-3-independent pathway. Thus, keratinocytes exposed to high-dose UVB stress possess at least two pathways for the induction of cell death.

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